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Collagen in the Spicule Organic Matrix of the Gorgonian *Leptogorgia virgulata*

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Abstract. Decalcification of the calcareous spicules from the gorgonian *Leptogorgia virgulata* reveals an organic matrix that may be divided into water insoluble and soluble fractions. The insoluble fraction displays characteristics typical of collagen, which is an unusual component of an invertebrate calcium carbonate structure. This matrix fraction exhibits a collagenous amino acid profile and behavior upon SDS-PAGE. Furthermore, the reducible crosslink, dihydroxylysinonorleucine (DHLNL), is detected in this fraction. The composition of the matrix varies seasonally; *i.e.*, the collagenous composition is most prevalent in the summer. These results indicate that the insoluble matrix is a dynamic structure. Potential roles of this matrix in spicule calcification are discussed.

Introduction

The mesoglea of the gorgonian *Leptogorgia virgulata* contains microscopic calcite (calcium carbonate) spicules (Kingsley and Watabe, 1982). Isolation and decalcification of the spicules yield an organic matrix, which is intimately involved in calcification (see Wilbur and Simkiss, 1968; Weiner and Traub, 1981; Watabe, 1981).

Unlike vertebrate osseous tissues that consist of hydroxyapatite (calcium phosphate) and collagen, collagen has not been associated with the formation of invertebrate calcium carbonate structures (Jope, 1967; Watabe, 1981; Benson *et al.*, 1983; Swift *et al.*, 1986). The presence of collagen, or a collagen-like component, in the spicule proteins of gorgonians had been suggested previously (Silberberg *et al.*, 1972; Goldberg, 1988), but this could not be confirmed (Kingsley and Watabe, 1983).

This report presents conclusive evidence for a predominant, insoluble "classic" collagen component within isolated and apparently homogeneous calcite spicules of *L. virgulata* collected in the summer months. The collagen is partially characterized, and noncollagenous components of the insoluble matrix are examined as well. Potential roles of the insoluble matrix in spicule calcification are discussed.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PAS, periodic acid-Schiff's reagent; TES, N-Tris-(hydroxymethyl) methyl-2-aminoethane sulfonic acid; DHLNL, dihydroxylysinonorleucine.

Materials and Methods

Colonies of the gorgonian *Leptogorgia virgulata* were collected at low tide from Sixty Bass Creek of North Inlet Estuary, Georgetown, South Carolina, in the summer of 1985 and from the subtidal waters off Morehead City, North Carolina, in March, July, and December of 1987. Colonies were immediately cleaned of adhering organisms and debris, frozen, and transported on dry ice and stored at −30°C.

Organic matrix preparation

All preparations were conducted at 4°C unless otherwise indicated. The tissues of the colonies that contain the spicules were stripped from their axes, weighed, and washed with 0.02 *M* NH_4HCO_3. The tissue was suspended in 10 volumes of 0.25 *M* NaCl in 0.2 *M* NH_4HCO_3 buffer adjusted to pH 8.0. The spicules remained insoluble under these conditions. They were released from their surrounding tissues by digestion (24 h, at 37°C, with shaking)

with 1% papain (substrate/enzyme, w/w) activated with 0.005 *M* cysteine. The supernatant was decanted, and any undigested tissue was treated with additional papain, as described above, for another 24 h. The tissues surrounding the spicules were completely digested following these treatments. The remaining spicules were washed thoroughly with the above buffer and retained on a 250 μm mesh sieve. The spicules, while contained in the sieve, were washed thoroughly with the same buffer to avoid any possible contamination by enzyme or solubilized material. Examination under a microscope indicated an apparently homogeneous preparation of spicules.

Spicules were suspended in an equal volume of 0.5 *M* potassium EDTA in 0.05 *M* NH_4HCO_3, pH 8.0, and demineralized by dialysis (with 3500 dalton cut off tubing) against the same solution. Following demineralization, the total content was recovered from the dialysis tubing and centrifuged. The insoluble organic matrix residue was washed 3 times with 0.2 *M* NH_4HCO_3, 6 times with 0.05 *M* NH_4HCO_3, and 3 times with distilled water. The washed insoluble matrix was lyophilized. The supernatant and the washings, which contained the soluble matrix proteins, were dialyzed exhaustively against distilled water (same cut off tubing), and then lyophilized.

Amino acid analysis

The amino acid compositions of: (1) total insoluble matrices from South Carolina, and March, July and December samples from North Carolina; (2) subfractions of the North Carolina July insoluble matrix (described below); and (3) the soluble matrices from July and December were determined. Each sample was hydrolyzed in 200 μl of 6 *N* HCl in an N_2 atmosphere, for 20 h at 110°C. Amino acid analysis was performed on a Varian 5560 Liquid Chromatograph using a stainless steel cation-exchange column (0.4 × 25 cm, AA 911, Interaction) with post column ninhydrin detection. Color was developed at 135°C in a stainless steel reaction coil (Yamauchi *et al.*, 1986).

Collagen characterization

The insoluble matrix was treated with 5% pepsin (w/w) in 10 volumes of 0.5 *M* acetic acid for three days at 21°C. The reaction mixture was centrifuged at 25,000 RPM in an ultracentrifuge for 1 h. The insoluble portion was washed thoroughly with 0.5 *M* acetic acid and lyophilized. The supernatant was brought to 3.0 *M* NaCl in the cold and allowed to stand overnight. The precipitate, after centrifugation at 10,000 RPM for 20 min, was redissolved in 0.1 *M* acetic acid, exhaustively dialyzed against 0.1 *M* acetic acid, and lyophilized. The supernatant of the 3.0 *M* NaCl solution was dialyzed exhaustively against distilled water, and lyophilized. No material was observed. The original insoluble matrix, pepsin solubilized material and pepsin-insoluble material were hydrolyzed as described above and subjected to amino acid analysis.

Cyanogen bromide (CNBr) cleavage

The various fractions obtained above were treated with 25% mercaptoethanol in 0.2 *M* NH_4HCO_3 at 55°C overnight to completely reduce any methionine sulfoxide residues back to Met (methionine). These fractions were then lyophilized, digested with CNBr in 70% formic acid in an N_2 atmosphere for 4 h at room temperature, diluted with distilled water, and lyophilized again.

Polyacrylamide gel electrophoresis (PAGE)

Portions of each fraction, both treated and untreated with CNBr, were subjected to PAGE in 0.1% SDS (Laemmli and Favre, 1973). Soluble type I collagen from foetal bovine skin was used as a standard. Tube gels (4% acrylamide) and gradient slab gels (3–17% acrylamide) were employed. Some of the very insoluble samples were treated at 100°C for 10 min with 0.2% SDS electrode buffer (Laemmli and Favre, 1973) to which 2 *M* urea was added. Gels were stained for protein with 0.05% Coomassie Brilliant Blue, and for carbohydrates with the PAS reagent (Zacharius *et al.*, 1969).

Determination of cross-links

Samples of lyophilized untreated insoluble spicule matrix were suspended in 0.15 *M* TES buffer, pH 7.5, and reduced with standardized NaB^3H_4 (Fukae and Mechanic 1980; Yamauchi *et al.*, 1986). The reduced insoluble matrix was hydrolyzed *in vacuo* with 3 *N* HCl for 48 h at 115°C (Yamauchi *et al.*, 1986). Analysis of cross-links was performed on a Varian 5560 Liquid Chomatograph as described previously (Yamauchi *et al.*, 1986). Hydroxyproline (Hyp) analysis was performed by amino acid analysis and residues of cross-link per mole of collagen was calculated on the basis of 300 residues of Hyp per molecule collagen.

Results

Amino acid compositions

The amino acid compositions of the matrix fractions of gorgonians collected from South Carolina and North Carolina are shown in Table I. Also presented are amino acid compositions of an invertebrate collagen and a mammalian mineralized collagen (Table I I, J, respectively). The compositions of the insoluble matrices of the two summer samples are similar (Table I A, C). Both samples display compositions typical of a collagen, since they contain significant amounts of Hyp, Hyl (hydroxy-

TABLE I

Amino acid compositions of matrix protein fractions from isolated calcite spicules of Leptogorgia virgulata

	Residues per 1000 Total Residues									
	A TIM S.C.	*B* TIM 3/87	*C* TIM 7/87	*D* TIM 12/87	*E* Pepsin sol	*F* Pepsin res	*G* SM 12/87	*H* SM 7/87	*I* Sea anemone collagen[d]	*J* Bovine bone[e]
Hyp	74	8	82	31	80	11	1	—	103	98
Asp	93	132	76	130	84	153	516	577	73	45
Thr[a]	36	57	34	48	32	63	18	16	37	17
Ser[a]	43	57	42	53	43	78	16	13	41	34
Glu	109	87	94	92	112	76	34	32	97	74
Pro	66	67	65	59	64	50	17	19	67	123
Gly	295	158	332	225	335	161	210	185	339	337
Ala	83	78	82	76	77	67	130	108	65	109
Val	27	58	26	37	22	55	17	16	25	20
Cys[b]/2	2	16	1	10	—	17	—	—	—	—
Met[c]	4	11	7	10	5	10	—	—	—	5
Ile	18	38	15	26	13	46	6	5	22	11
Leu	27	53	26	42	23	58	7	6	30	25
Tyr	8	24	8	20	6	32	—	2	3	4
Phe	9	31	9	22	7	35	4	4	8	13
His	3	17	4	11	3	11	2	1	1	4
Hyl	33	7	34	15	34	6	—	—	25	6
Lys	15	44	12	33	10	33	12	9	16	26
Arg	56	55	50	53	50	39	8	5	68	50

TIM—Total Insoluble Matrix.
S.C.—Summer collection in South Carolina.
3/87, 7/87, 12/87—Dates of collection in North Carolina.
Pepsin sol—7/87 collection, pepsin soluble material.
Pepsin res—7/87 collection, pepsin insoluble material.
SM—Soluble Matrix.
[a] Uncorrected for hydrolysis.
[b] Half Cys, sum of cysteic acid and cystine.
[c] Sum of methionine sulphoxide and methionine.
[d] Nowack and Nordwig 1974.
[e] Herring 1972.

lysine) and 33% Gly (glycine). The composition in column C is extremely close to that of the pepsin solubilized invertebrate collagen of the sea anemone (column I). The amino acid composition of the insoluble matrix from samples collected in March 1987 displays markedly lower values of Hyp, Hyl and Gly (Table IB). Gly, Asp (aspartic acid) and Glu (glutamic acid) are the most abundant amino acids. The insoluble matrix from samples collected in December 1987 displays an amino acid composition that is intermediate to the March and July compositions (Table ID).

The July insoluble spicule matrix, which was partially solubilized by pepsin digestion in 0.5 *M* acetic acid and precipitated by addition of 3.0 *M* NaCl, also has the composition typical of a collagen (Table IE). This precipitated collagen is a white fibrous material that displays an amino acid composition similar to that of the whole insoluble matrix (Table IC) and sea anemone collagen (Table II). The amino acid composition of the fraction of the July insoluble matrix that is not soluble in pepsin and acetic acid (Table IF) is similar to that of the insoluble matrix of the spicules collected in March (Table IB).

The soluble matrix fractions are off-white and extremely hygroscopic. Samples from December and July display similar amino acid compositions. The most prominent feature of this fraction is its extremely high aspartic acid content (>50%).

PAGE

The pepsin solubilized spicule collagen was subjected to 4% acrylamide SDS-PAGE and compared to type I bovine soluble skin collagen (Fig. 1). Although the bands of the spicule matrix are faint, they can be seen to travel in the range of type I collagen. The spicule collagen contains a component similar in mobility to the α1 (I) chain.

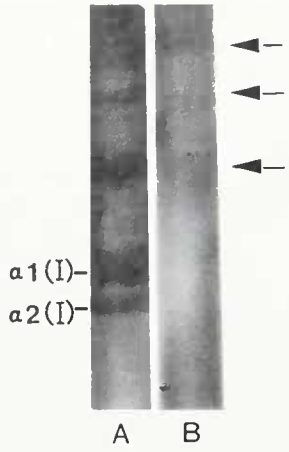

Figure 1. SDS-PAGE of (A) type I collagen, and (B) the total insoluble matrix of *Leptogorgia virgulata*

Also present are molecular weight components equal to the β and γ chains of type I collagen.

A CNBr digest of the solubilized collagen from the July insoluble matrix showed, on 3–17% acrylamide gradient gels, different patterns from that of a CNBr digest of type I collagen (Fig. 2). Treatment with urea did not change the pattern of the insoluble matrix. The total insoluble matrix apparently did not enter the gel and therefore remained uncleaved by CNBr.

SDS-PAGE of the matrix not solubilized with pepsin revealed low molecular weight proteins that stained with PAS (Fig. 3), indicating the presence of glycoproteins in this matrix.

Following the NaB^3H_4 reduction of the insoluble matrix, cross-link analysis indicated that the only reduced cross-link present was DHLNL (Fig. 4). Each nmole of collagen contained 2.87 nmoles of DHLNL. No stable non-reducible cross-links were detected.

Discussion

This report is the first to demonstrate conclusively the presence of a "classic" collagen (*i.e.*, a protein containing Hyp, Hyl, and 33% Gly as well as a typical collagen cross-link) as part of the organic matrix of a calcite (calcium carbonate) invertebrate skeletal structure. The insoluble organic matrices from calcite spicules of *Leptogorgia virgulata* collected in the summer months have amino acid compositions characteristic of collagen (Table IA, C). Those compositions are similar to other soft tissue coelenterate collagens (Franc, 1985). When the amino acid composition is viewed *in toto*, strong similarities to the pepsin soluble fraction, and sea anemone and vertebrate collagen are evident (see Table I C, E, I, J). The SDS-PAGE pattern of the July insoluble matrix (after pepsin digestion) also displayed the characteristic of type I collagen (100,000–300,000 daltons). However, the *Leptogorgia* matrices do not exhibit periodic bandings of typical vertebrate collagens (Watabe and Kingsley, unpub.).

The intermolecular reducible cross-link, dihydroxylysinonorleucine (DHLNL), was clearly detected in the collagen of the July insoluble matrix. Little if any other reducible and non-reducible cross-links were present. Similar observations have been made in other invertebrate collagens (Shadwick, 1985) including coelenterates (Bailey, 1971). The latter were from non-mineralized tissues. DHLNL is the most prevalent reducible cross-link in the type I collagen of bovine tendon, bone, and dentin (Mechanic *et al.*, 1971). Mechanic *et al.* (1985) have demonstrated that DHLNL is distributed in different molecular locations in these three tissues and may determine the functional properties of collagen. They suggest that the presence of multifunctional cross-links (*i.e.*, histidinohydroxylysinonorleucine, pyridinoline, and histidinohydroxymerodesmosine) in the nonmineralized collagen tissues holds the molecules at a shorter distance than do the bifunctional cross-links of the mineralized collagen in bone, thereby physically precluding the entrance of ions and the subsequent formation of hydroxyapatite crystals. Conversely, once bone collagen is calcified, the mineral does not allow close enough juxtaposition of the molecules to form multifunctional cross-links (Mechanic *et al.*, 1985). The molecular location of DHLNL is not known in the spicule matrix of gorgonians, however, consistent with this theory, multifunctional cross-links are not present.

We have used enzymic digestion by papain at pH 8.0 at 37°C in order to isolate a homogeneous population of calcite spicules. The mineral contained in the collagen of a mineralized tissue protects collagen from denaturation, as well as from enzymatic degradation at neutral pH and

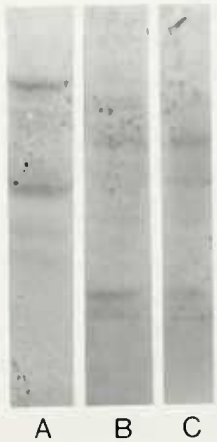

Figure 2. Cyanogen bromide cleavage patterns of (A) type I collagen; (B) pepsin solubilized insoluble matrix; and (C) pepsin solubilized insoluble matrix treated with urea.

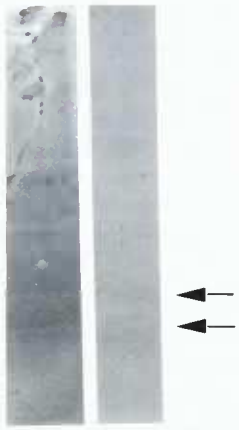

Figure 3. SDS-PAGE of the fraction of the insoluble matrix not solubilized in acetic acid and pepsin, stained for (A) protein with Commassie blue, and (B) carbohydrate with PAS.

above (Bonar and Glimcher, 1970). The physiological, relatively gentle digestion procedure used in this study degraded and solubilized all the collagenous and non-collagenous protein that was not protected by mineral. Previous methods used to isolate "calcified" structures used NaOCl or a strong base to destroy organic material. However, NaOCl will produce anorganic bone from normal mammalian bone and is often used to examine bone architecture by scanning electron microscopy. This also degrades as well as destroys a significant portion of the organic matrix of mineralized tissue. The above analysis is substantiated by a comparison of the current results with a previous failure to detect collagen in the insoluble matrix fraction (Kingsley and Watabe, 1983). Kingsley and Watabe (1983) isolated spicules in 5.25% NaOCl and demineralized in 0.1 *N* HCl at room temperature. The isolated spicules that were analyzed were, in both cases, from animals collected during the summer, so the previous methods were harsh and yielded erroneous results.

The spicule insoluble matrix of the gorgonian *Briareum asbestinum* consisted of very small peptides (1600–5000 daltons) and contained 22 Hyp residues/1000, approximately 20% Gly and no Hyl (Silberberg *et al.*, 1972). These features only suggest the presence of collagen-like components. The amino acid composition of the whole spicule matrix of the gorgonian *Pseudoplexaura flagellosa* revealed 48, 237, and 9 residues/1000 total residues of Hyp, Gly, and Hyl, respectively (Goldberg, 1988). Silberberg *et al.* (1972) air dried, ground the whole animals, digested the organic material with 30% KOH, and demineralized in concentrated HCl at 4°C. These procedures are extremely severe in the processing of biological material and may have resulted in the loss of collagenous components. The isolation of spicules in 1 *N* NaOH by Goldberg (1988) may have caused similar problems. However, such differences in the matrix compositions of the gorgonian species may also be the results of other factors including species variations, and the season and the environment from which specimens were collected (see below).

The results of the present study indicate that the amount of collagen in the insoluble matrix is directly related to the season of collection and, therefore, to temperature or other environmental conditions. The matrices isolated from animals collected in the summer from both South Carolina and North Carolina display Hyp and Gly levels similar to those of vertebrate collagen, as well as large amounts of Hyl (Table I A, C). Matrices from animals collected in March and December are different from the above; *i.e.*, they are less collagen-like in composition. In December, both the Hyp and Hyl contents were less than half of those of summer samples, and the Gly was reduced to 225 residues/1000. By March, the Hyp, Hyl, and Gly contents continued their reduction to 8, 7, and 158 residues/1000, respectively. These results clearly indicate a seasonal variation in the insoluble matrix. Had the analyses of the organic matrix compositions only been conducted on specimens collected in March, the predominance of the collagenous component of the matrix might

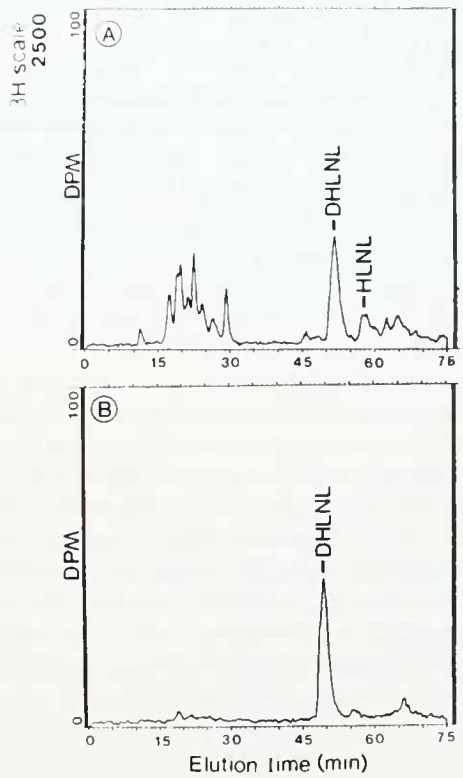

Figure 4. Elution patterns of reduced collagen from (A) bovine bone and (B) *Leptogorgia virgulata* spicule insoluble matrix. The positions of the intermolecular cross-links dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL) are shown.

have been overlooked. The seasonal changes in the insoluble matrix are not simply shifts of amino acid residues from insoluble to soluble fractions, because the soluble fractions in summer and winter have relatively similar amino acid compositions (Table I G, H).

The apparent seasonal variation suggests a remodeling or turnover of the collagen component, and thus a degree of demineralization and remineralization (turnover) in the spicules. A comparison of the amino acid composition of the non-solubilized portion of the insoluble matrix and the total insoluble matrix collected in March reveals strong similarities. If indeed there is partial demineralization of spicules in winter months, and the portion of the collagen that is pepsin soluble is lost, then the amino acid composition of the winter matrix and that of the non-solubilized fraction of the summer matrix should be similar. Certainly, in the remodeling of vertebrate bone, demineralization must occur prior to degradation of collagen. Spicules in *L. virgulata* are initially formed intracellularly, but later, they become exposed to the extracellular environment (Kingsley and Watabe, 1982). It is not clear whether spicule growth and maturation continues once these structures are in the extracellular environment, although this apparently occurs in other gorgonian species (Goldberg and Benayahu, 1987). The present results indicate that the spicules are dynamic structures even after they emerge from the cell.

The pepsin solubilized insoluble matrix shows a collagenous composition similar to total insoluble matrix. However, CNBr digestion of this solubilized collagen produced peptide patterns distinct from bovine skin type I collagen. Samples of the total insoluble matrix treated with CNBr remained insoluble even after reduction of any methionine sulphoxide to Met with 25% mercaptoethanol. No material was found to enter the gel. It is obvious that another insoluble protein is present that protects the collagen from digestion by CNBr.

The fraction of the insoluble matrix that was not solubilized by pepsin did not display a typical collagenous amino acid composition. Because it did contain some Hyp and Hyl, however, a portion of the collagen in the matrix is probably blocked from enzymic digestion. This predominantly non-collagenous matrix fraction on SDS-PAGE revealed low molecular weight glycoproteins, as is seen by Coomassie Blue and PAS staining. The presence of carbohydrates in invertebrate calcifying matrices is common and has been described previously in gorgonians (Kingsley and Watabe, 1983; Goldberg, 1988). The possibility that glycoproteins play a role in calcification has been proposed for several organisms, (see Crenshaw, 1972; deJong *et al.*, 1976; Fichtinger-Schepman *et al.*, 1979; Marsh and Sass, 1984).

The roles of collagen in biomineralization have been examined extensively in bones and teeth and may now be extended to the invertebrates. Some of the major theories of how collagen may be involved in the regulation of calcification involve its association with other non-collagenous components such as phosphate and osteonectin (see Prockop and Williams, 1982). Similarly, in *L. virgulata* the structure-functional relationship between the non-collagenous matrix and the insoluble matrix, may determine how the total organic matrix regulates spicule formation. Both Kingsley and Watabe (1983) and the present report indicate that the soluble spicule matrix of *L. virgulata* has a soluble acidic protein containing more than 50% Asp. Much of the calcium binding capacity of invertebrate mineralizing matrices has been attributed to the soluble fraction (see Watabe and Kingsley, 1989).

Although this is the first conclusive report of the presence of collagen within the organic matrix of a calcium carbonate skeletal structure, collagen has been found to be associated, indirectly, with a number of calcium carbonate structures. In the echinoderms, collagen is not found within the sea urchin larval spicule matrix (Blankenship and Benson, 1984; Wilt *et al.*, 1985); however, collagen metabolism is critical for normal spicule formation. Collagen is apparently necessary for providing a permissive substratum in which spicule formation may occur (Blankenship and Benson, 1984). Similarly in the pennatulids, another coelenterate, collagen is closely associated with the calcitic crystals but is not responsible for the nucleation of the mineral (Ledger and Franc, 1978). Other such indirect roles of collagen in invertebrate calcification are discussed by Watabe and Dunkelberger (1979) and Kingsley (1984).

Acknowledgments

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